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Effects of glycine, β-alanine and diazepam upon morphine-tolerant-dependent mice*

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The effects in mice of glycine, β-alanine and diazepam on the analgesic response to morphine, on the intensity of tolerance and on the physical dependence on the analgesic have been examined. The two amino acids increased the analgesic response to morphine in a dose-related manner. However, both compounds were ineffective in the analgesic test (hot plate) when administered without morphine. Diazepam was ineffective in the analgesic test and it did not alter morphine analgesia, except when administered in a high dose which decreased and analgesic response. Glycine, either in single or repeated doses, did not modify tolerance to morphine, whereas β-alanine induced a dose-related partial antagonism, which promptly reached a plateau. Diazepam induced a small decrease in the intensity of tolerance to the analgesic. The abstinence syndrome to morphine, induced by naloxone administration to primed mice, was reduced by single doses of glycine or β-alanine. Diazepam behaved as a weak inhibitor of the abstinence syndrome when administered at a high dose. The potentiation of morphine analgesia and the antagonism of the abstinence syndrome induced by the amino acids may be related to their hyperpolarizing action in the c.n. system. The effects of β-alanine on morphine tolerance cannot be explained by the same mechanism.

Glycine is one of the major inhibitory neurotransmitters of the central nervous system (c.n.s.) (Triggle & Triggle 1976; DeFeudis 1978). Its inhibitory actions are exerted on interneurons of the spinal cord and brain stem and some neurons of higher centres (Aprison et al 1970; Snyder 1975). The interaction of morphine and glycine has been studied by means of their iontophoretic administration on neurons of the spinal cord and other neurons of the c.n.s. of the cat (Dostrovsky & Pomeranz 1973; Duggan et al 1976). These electrophysiological studies have produced controversial results, antagonistic and synergistic effects of morphine on the depressant action of glycine having been reported (Duggan et al 1976).

The purpose of this work was to study the effects of the administration of glycine on some morphine actions in the mouse. The influence of the amino acid on the effects of morphine was assessed on analgesia, tolerance and dependence. The possible interaction of other compounds that have been shown to bind to glycine receptors of the c.n.s. was also examined. Thus, β-alanine and diazepam were given to mice under acute or continued morphine administration.

METHODS AND MATERIALS

Adult female mice from a strain raised at our department were maintained under standardized conditions of light (800–1800 h), temperature (22 ± 2 °C) and food. The drugs were morphine hydrochloride (May & Baker), glycine (Sigma), β-alanine (Sigma), diazepam (Hoffman-LaRoche) and naloxone hydrochloride (gift from Endo Labs. Inc.).

Analgesia

Analgesia was assessed by the hot plate procedure (Eddy & Leimbach 1953). Mice were dropped on the surface of a copper container maintained at 55 ± 0·5 °C by a thermoregulated water-circulating pump. The responses consisted of jumping off the plate or kicking the legs. Each animal was tested twice before drug administration and the values recorded were averaged to obtain a baseline. The effects of the amino acids and diazepam administered without morphine were similarly studied. Reaction times were determined 30 min after the administration of morphine, and thereafter every 30 min for 2 h. Glycine, β-alanine or diazepam was given 45 min before the test dose of analgesic. The total analgesic response was calculated from the area under the time-response curve (Winter & Flataker 1950).

Induction of tolerance and physical dependence

Tolerance and dependence were induced by a slow release preparation of morphine of the following composition: morphine (as free base) 300 mg, sorbital sesquioleate 0·80 ml, liquid paraffin 4·20 ml

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and 0·9% NaCl (saline) 5·00 ml. Each mouse received 300 mg kg^{-1} s.c. of morphine.

The degree of tolerance was expressed as the ratio of the mean analgesic effect of a test dose of morphine in naive and in primed mice. The antagonism or attenuation of tolerance was determined by the statistical difference between the effects of the test dose in primed untreated animals and those observed in primed treated mice. A level of probability of 0·01 was accepted as significant. This criterion was weighed by the requirement that a decrease of at least 3 units in the degree of tolerance had to be observed between the values of the treated and the primed untreated groups. The observed value in primed untreated mice was 6·8. This value is near the maximal intensity of tolerance discernible by the method employed, since the reponses in the primed mice were similar to the responses of the saline controls. For this reason, in those groups of mice in which the administered compound did not intensify the response observed in the primed untreated mice, the degree of tolerance was expressed as $> 6{\cdot}8$.

A comparison of the percent change in analgesic response was also made after the administration of the compounds assayed with morphine.

Naloxone precipitated abstinence

After 30 h of the slow-release administration of morphine, mice were injected with a dose of naloxone (4 mg kg^{-1}) that produced signs of severe abstinence such as diarrhoea, body shakes, paw tremors, abnormal posturing and jumping behaviour (Contreras et al 1977, 1979). The number of mice that showed these signs in a 20 min observation period was recorded. The Chi^2 test was used for statistical analysis of the differences between the control (untreated) and treated mice. A level of probability of 0·05 was accepted as significant.

RESULTS

Analgesia and tolerance

Hot plate reaction times were unaltered after the i.p. injection of glycine (80, 100 and 120 mg kg^{-1}), β-alanine (100, 125 and 150 mg kg^{-1}) or diazepam (1, 2 and 3 mg kg^{-1}).

Although glycine was ineffective when administered alone, it significantly increased the acute effects of a test dose of morphine. This synergistic action of the amino acid was dose-related (Table 1).

Glycine had no effect on the degree of tolerance when single doses were injected 45 min before the test dose of morphine. As shown in Table 1, the amino acid did not modify the response to the analgesic compared with the results observed in primed untreated mice. To avoid the interpretation that the amino acid is increasing tolerance to the analgesic, the degree of tolerance is expressed $>6{\cdot}8$. A group of mice was treated with 5 doses of glycine of 80 mg kg^{-1} each, the last being given 45 min before the test dose of morphine. This treatment did not alter the degree of tolerance compared with that in the groups receiving a single dose of the amino acid.

Table 1. Effects of glycine on morphine tolerance in mice.

Experimental condition	Analgesic response to morphine, 5 mg kg^{-1} s.c., in mice (hot plate method). Area under the time response curve ± s.e.m.				
	Naive	% of change of analgesia	Primed	% of change of analgesia	Degree of tolerance[1]
Saline	62 ± 26		65 ± 42		
Morphine (untreated)	504 ± 28		74 ± 10		6·8
Morphine Glycine 80 mg kg^{-1}	650[2] ± 34	29	71 ± 29	−5	>6·8
Morphine Glycine 100 mg kg^{-1}	710[2] ± 36	41	78 ± 32	5	>6·8
Morphine Glycine 120 mg kg^{-1}	822[2] ± 42	63	73 ± 30	−2	>6·8
Morphine Glycine 80 mg kg^{-1} 5 doses[3]	1208[2] ± 50	140	70 ± 30	−6	>6·8

[1] Ratio of the mean analgesic effects to the test dose in naive and in primed mice.
[2] Significantly higher than the effects of morphine in naive untreated mice. $P < 0{\cdot}01$.
[3] Glycine was administered at 830, 1200, 1430 and 1830 during the first day. The last dose of glycine was injected 45 min before the test dose of morphine.

The administration of β-alanine in combination with morphine induced similar acute results to those of glycine, i.e. a dose-related increase in the analgesic effect of morphine (Table 2). β-Alanine differed from glycine when administered to tolerant mice since it decreased the degree of tolerance in the primed animals. The higher values were statistically significant and consistent with the criterion of a decrease of at least 3 units in the degree of tolerance in order to accept an attenuation of tolerance. The attenuating effect on tolerance seemed to reach a maximum as can be observed from the results obtained by repeated treatment with β-alanine during the course of tolerance (Table 2).

Diazepam did not increase the acute effect of the test dose of morphine, but a high dose (7·5 mg kg^{-1}) decreased this effect (Table 3). In the group of mice receiving 3 mg kg^{-1} diazepam, the degree of tolerance was attenuated (Table 3).

Table 2. Effects of β-alanine on morphine tolerance in mice.

Experimental condition	Analgesic response to morphine, 5 mg kg^{-1}, in mice (hot plate method) Area under the time response curve ± s.e.m.				
	Naive	% of change of analgesia	Primed	% of change of analgesia	Degree of tolerance[1]
Morphine (untreated)	504 ± 28		74 ± 10		6·8
Morphine β-alanine 100 mg kg^{-1}	734[2] ± 70	45	274[3] ± 55	270	2·6
Morphine β-alanine 125 mg kg^{-1}	882[2] ± 78	75	355[3] ± 28	379	2·4
Morphine β-alanine 150 mg kg^{-1}	1146[2] ± 84	127	434[3] ± 27	486	2·6
Morphine β-alanine 100 mg kg^{-1} 5 doses[4]	902[2] ± 84	79	408[3] ± 58	441	1·7

[1] Ratio of the mean analgesic effects to the test dose in naive and in primed mice.
[2] Significantly higher than the effects of morphine in naive untreated mice. $P < 0.01$.
[3] Significantly higher than the effects of morphine in primed untreated mice. $P < 0.01$.
[4] β-alanine was administered at 830, 1200, 1400 and 1830 during the first day. The last dose of β-alanine was injected 45 min before the test dose of morphine.

Abstinence syndrome

The abstinence behaviour, evoked by naloxone, was reduced by the prior administration of glycine and β-alanine (Table 4). Glycine at a dose of 80 mg kg^{-1} significantly attenuated the frequency of animals presenting positive responses in 5 of the 6 signs registered. The administration of several doses of the amino acid during the course of dependence, failed to increase its attenuating effect.

Table 3. Effects of diazepam on morphine tolerance in mice.

Experimental condition	Analgesic response to morphine, 5 mg kg^{-1}, in mice (hot plate method) Area under the time response curve ± s.e.m.				
	Naive	% of change of analgesia	Primed	% of change of analgesia	Degree of tolerance[1]
Morphine (untreated)	504 ± 28		74 ± 10		6·8
Morphine diazepam 1 mg kg^{-1}	572 ± 63	13	77 ± 23	4	>6·8
Morphine diazepam 2 mg kg^{-1}	530 ± 44	5	97 ± 37	31	5·4
Morphine diazepam 3 mg kg^{-1}	560 ± 68	11	190[2] ± 16	156	2·9
Morphine diazepam[4] 7·5 mg kg^{-1}	396[3] ± 27	−22	73 ± 32	−2	5·4

[1] Ratio of the mean analgesic effects to the test dose in naive and in primed mice.
[2] Significantly higher than the effects of morphine in primed untreated mice. $P < 0.01$.
[3] Significantly lower than the effect of morphine in naive untreated mice $P < 0.02$.
[4] Diazepam was administered in two doses, 5 mg kg^{-1} simultaneously with the priming dose of the analgesic and 2·5 mg 10 h later.

β-Alanine was effective in reducing the frequency of two abstinence signs body shakes and jumping. Its attenuating effects were not enhanced by its administration during the course of dependence.

Single doses of diazepam had no effect on the abstinence behaviour. When the drug was administered during the course of dependence a decrease in the number of jumps was observed. The other signs remained unchanged.

DISCUSSION

Our results clearly show that glycine has a synergistic influence on the acute effect of morphine. The potentiating effects observed in morphine analgesia seem to be unspecific for the analgesic used, since the amino acid also increases the sleeping time induced by hexobarbitone (unpublished results). Moreover, Chan (1978) reported that glycine also enhances the ethanol sleeping time when both are simultaneously injected in mice. This latter effect is produced without significant changes in the amino acid concentration in the brain and without altering the metabolism of ethanol.

The iontophoretic administration of glycine produces membrane hyperpolarization which is thought to be mediated by an increase in Cl^- conductance (DeFeudis 1978). Most pharmacological studies have been made by directly placing the amino acid on neurons of the c.n.s. Morphine, on the other hand, antagonizes the depressant effect of glycine on spinal neurons of the cat (Dostrovsky & Pomeranz 1973). In contrast, Duggan et al (1976) have described synergistic and antagonistic actions of morphine and glycine in several interneurons of the c.n.s. These results are difficult to interpret due to the complex organization of the neurons of the c.n.s. Our data suggest that glycine is an effective agent in increasing acute morphine action and in attenuating the abstinence behaviour induced by morphine deprivation. The synergistic effect with morphine is dose-related. However, the amino acid did not alter the tolerance to morphine even when several doses were administered during tolerance development.

Benzodiazepines have been shown to interact, at least partially, with glycine receptors (Snyder 1975; Young et al 1974). Furthermore, these drugs have also been mentioned in relation to narcotic analgesics (Shannon & Holtzman 1976; Shannon et al 1976). Thus, we have examined their influences on those effects of morphine which were modified by the inhibitory amino acid; diazepam was used for this purpose. In general, the effects of diazepam differed from those of glycine. The drug did not increase the

Table 4. Effects of glycine, β-alanine and diazepam on the abstinence syndrome to morphine (1).

Abstinence sign	Saline	Glycine 40 40 mg kg^{-1} 30 min before naloxone	Glycine 80 80 mg kg^{-1} 30 min before naloxone	Glycine during the course of depen-dence[2]	β-Alanine 100 mg kg^{-1} 30 min before naloxone	β-Alanine 150 mg kg^{-1} 30 min before naloxone	β-Alanine during the course of depen-dence[3]	Diazepam 1 mg kg^{-1} 30 min before naloxone	Diazepam 2 mg kg^{-1} 30 min before naloxone	Diazepam during the course of depen-dence[4]
Restlessness	18/18	14/18	9/18*	16/16	13/14	12/14	13/14	14/14	12/14	14/14
Abnormal posturing	18/18	14/18	16/18	14/16	12/14	13/14	12/14	12/14	14/14	14/14
Paw tremors	18/18	12/18*	9/18*	7/16*	12/14	13/14	13/14	12/14	12/14	12/14
Body shakes	17/18	7/18*	3/18*	4/16	5/14*	5/14*	4/14*	14/14	13/14	12/14
Jumping	16/18	4/18*	2/18*	0/16*	3/14*	4/14*	4/14*	12/14	12/14	0/14*
Diarrhoea	18/18	8/18*	4/18*	4/16*	13/14	10/14	14/14	13/14	13/14	12/14

[1] Induced by naloxone, 4 mg kg^{-1} i.p., 30 h after the administration of a sustained release preparation of morphine (300 mg kg^{-1} s.c.)
[2] Glycine, 80 mg kg^{-1} i.p. at 830, 1230, 1430 and 1830 of the first day of morphine administration and at 815 of the following day.
[3] β-Alanine, 100 mg kg^{-1} as indicated for [2].
[4] Diazepam 5 mg kg^{-1} i.p. at 830 and 2·5 mg kg^{-1} at 1830 of the first day of morphine administration. Morphine was administered at 900 h.
* Significantly different from those observed in saline-injected mice ($P < 0.05$).

analgesic response to a test dose of morphine, and when administered at high doses it attenuated the effect of the analgesic in the thermal stimulation. It also differed from glycine when it was given to tolerant mice. Diazepam produced a small but significative reduction of tolerance. The drug was also effective when administered during the course of dependence since it attenuated the abstinence behaviour. Diazepam behaved as a weak morphine antagonist, which is consistent with the results of Shannon et al (1976).

β-Alanine has been shown to be a ligand for glycine receptors (Snyder 1975; DeFeudis 1978; Triggle & Triggle 1976). For this reason the influence of this amino acid was also studied: in contrast to diazepam, β-alanine shared some effects of glycine: it increased the analgesia and reduced the intensity of the withdrawal syndrome. However, β-alanine differed from glycine in that it attenuated tolerance in a dose-related manner, but its maximal effect was obtained by a single dose and was not increased by sustained treatment.

Although glycine and β-alanine reduced the intensity of the abstinence behaviour, these effects cannot be ascribed only to the expression of physical dependence, since, both were as active when given during the induction of physical dependence as when administered a few minutes before the precipitation of abstinence. Their effectiveness may result from either a sensibilization of the nervous system to the depressant effects of morphine or to their hyperpolarizing effects in the nervous tissue.

The most remarkable effects of β-alanine was its ability to reduce the intensity of tolerance. This effect cannot be ascribed to a 'glycine-like' action through an occupancy of glycine receptors. β-alanine has also been shown to be effective in depressing the crayfish neuromuscular junction (Dudel 1965) in which the inhibitory response is produced by means of the occupancy of GABA receptors (Curtis 1963; Grundfest 1964). Consequently, it is possible that its inhibitory action on morphine tolerance might be explained by its action on the GABA mechanisms. This tentative explanation is supported by the observation that the decrease of tolerance is similar to the effects observed when an increase of GABA concentration in the c.n.s. is produced in mice (Contreras et al 1979).

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